Substance P in Long-Lasting Asthma

Immunoinflammatory pathways

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Background: Substance P (SP) was described at the beginning of the 20th Century, and its biological action was recognized to have implications in neurogenic inflammation and constriction of smooth muscles. The changes associated with inflammatory chronicity can compromise organ function reversibility. The role of neuromechanisms in the pathology of the disease has been investigated in order to achieve better diagnosis and therapeutic approaches. The stimulation of human cells, such as macrophages and polymorphonuclear cells by SP leads to their activation and to the release of reactive oxygen species (ROS) by these cells. Consequently, a continuous inflammatory disability is observed, mainly if a decrease in antioxidant defence occurs. SP is a substrate for dipeptidyl peptidase IV (DPPIV), which is a multifunctional molecule with enzymatic and proinflammatory activities. CD26 is considered an activation T cell marker. The aim of the present study was to analyse if serum SP values in long-lasting asthma patients were related to lung function parameters. It was also decided to analyze the relationship of SP with superoxide dismutase (SOD) and total antioxidant activity in serum (TAS), as well as its association to CD26/DPPIV values considering their immunological and inflammatory properties.

Methods/Data base: A group of individuals older than 65 years, including 64 asthmatic patients (mean age 72±5 years) and 41 healthy individuals (mean age 79±7years) was selected. Both subgroups were submitted to clinical observation, to skin prick tests (SPT) and to SP, TAS, SOD, and DPPIV determinations. T cell CD26 typing was also performed. Lung function tests were done on all patients.

Results: Among the patients studied, 42 had positive skin tests, mainly to house dust mites. Asthmatic patients showed a significant increase in SP values (116.2±138.9 vs 39.5±17.9 pg/ml) when compared to controls and a significant decrease in TAS levels (.85±.13 vs .91±.10 mM) and in SOD levels (588.1±156.1 vs 822.9±179.5 U/gHb). All patients were clinically stable and presented an average percentage of predict forced expiratory volume in the first second (FEV1) of 73.6±25.3 and median expiratory flow percentage of predict (MEF50) of 38.8±26.7. DPPIV values were significantly increased in asthmatics compared to controls (69.7±15.2 vs 58.6±14.3 U/L). The CD26 expression was only slightly increased in asthmatic patients (41.9±10.2 vs 39.4±11.4).

Conclusion: These results confirm the role of SP in asthma and give a contribution for a better knowledge of the immunoinflammatory pathway associated with this chronic disorder. A final goal for these studies would be to achieve a better therapeutic approach in order to improve the outcome of asthmatic patients.

Keywords: asthma, elderly, SP, DPPIV, SOD, TAS, CD26

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Introduction

Substance P (SP) was named by Gaddum and Schild, at the beginning of the 20th century, referring to the powder extracted from equine tissues, which had potent hypotensive and contractile properties. Later it was introduced as part of the tachykinins family, just as neurokinin A (NKA) and neurokinin B (NKB), which share the same carboxyl terminal sequence [1]. The biological actions of substance P are mediated by tachykinin receptors, which belong to rhodopsin-like membrane structures. There are three types of tachykinin receptors exhibiting substance P preferences for NK1 [1]. Some of the most considerable effects produced by substance P and other tachykinins released from peripheral endings of primary sensory neurons are collectively referred to as “neurogenic inflammation.” Responses produced at the peripheral level by sensory

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neuropeptides are prominent on the vasculature but additional tissue-specific responses such as bronchoconstriction have been recognized.

Asthma is a chronic inflammatory disorder of the airways, characterized by a widespread but variable bronchial obstruction and by hyperresponsiveness to several triggers [2]. The limitation to the airflow described depends on the addictive effect of the localized inflammatory process and on the smooth muscle contraction, which in part results from the tachykinin action. Asthmatic patients tend to develop a progressive decline in pulmonary function that is correlated with age, sex, duration and severity of the disease [3].

Lung function studies have demonstrated that the elderly population with long-lasting asthma presents increased basal airway narrowing and decreased bronchial reversibility more considerable than in older individuals with late onset asthma and disease evolution < 10 years [4, 5]. This respiratory function pattern can be even more severe as a consequence of the immunoinflammatory changes associated with the ageing process [6].

Recent studies with substance P antagonists have demonstrated that SP has various proinflammatory and spasmogenic effects both in asthma and in chronic bronchitis [1]. SP causes degranulation of mast cells through direct activation of plasma membrane G proteins mediating nonatopic hypersensitivity reactions [7]. SP can stimulate hematopoiesis and inflammatory leukocytosis. Lymphocytes, macrophages, and mast cells have SP receptors and can be stimulated to produce cytokines by SP. Once SP can also be produced by inflammatory cells, a positive feedback mechanism to the inflammatory process can be established through this tachykinin activity. The stimulation of human macrophages and polymorphonuclear cells by SP leads to reactive oxygen species (ROS) production and to interleukins and myeloperoxydase release [7–11]. These phenomena are more evident during asthma attacks with epithelial damage [12, 13]. To minimize the damage of oxidative products, cells are equipped with an extensive repertoire of antioxidant enzymes, like the superoxide dismutase (SDO). If a reduction of the antioxidative capacity occurs, disturbances in the oxidant/antioxidant imbalance can emerge [14].

Several enzymes are involved in the metabolism of SP, including neutral endopeptidase (NEP), angiotensin-converting enzyme (ACE), and dipeptidyl aminopeptide IV (DPPIV) [1, 15]. The T-cell activation antigen CD26 is recognized as the cellular marker of DPPIV, a membrane peptidase. DPPIV is a multifunctional molecule which, beside enzymatic activity, exhibits several important functions: It can act as an adhesive molecule by binding fibronectin, it can serve as a receptor for other enzymes, such as adenosine deaminase (ADA), it is the functional receiver of collagen, and it can act as a costimulatory molecule transducing the signal through CD3 on T lymphocytes. CD26 participates in cellular signalling due to its close association to membrane-linked protein-tyrosine phosphatase CD45, identified as the protein of surface of larger expression in the hematopoietic nuclear cells, resulting in the modulation of cellular proliferation [16]. CD26/DPPIV is highly expressed in a great variety of cells but in blood cells it is detected almost exclusively in activated T lymphocytes [17–21].

The aim of the present study was to analyze the behaviour of serum SP in long-lasting asthma, considering its role as an inflammatory and bronchoconstriction inductor, evaluating lung function parameters and establishing comparisons with SP values found in a control population of the same age. It was also decided to analyze the TAS and SOD production in the same population, as well as CD26/DPPIV, considering its role in cellular activation and modulation of the immunoinflammatory response.

Methods

Subjects

A group of 105 nonsmoking elderly individuals (> 65 years, range 65–94 years) was selected after informed oral consent.

Baseline screening data of 64 patients with mild persistent asthma were used in this evaluation. All patients had a history of intermittent chest tightness, wheezing or shortness of breath for at least 30 years prior to participation in the study consistent with the diagnosis of asthma according to the Global Initiative for Asthma (GINA). All subjects had asthma, controlled by using 250–500μg of beclometasone dipropionate daily and short-acting β2-agonists as needed. All other antiasthmatic drugs were withdrawn at least 4 weeks prior to the study.

The control group included 41 individuals.

None of the participants had any respiratory infection in the month previous to the study. No other clinically relevant diseases were reported. Table 1 shows the characteristics of the individuals in both groups group. Sex distribution and age were similar in the two groups studied. Cancer, autoimmunity, infection, diabetes, heart failure, renal failure, chronic hepatic, or recent exposure the environmental risk factors were considered exclusion criteria.

Diagnosis Tests

All subjects were submitted to skin prick tests to the following 20 common aeroallergens (ALK-ABELLO; 1mm Prick Lancetter-tames Hollister Stier): *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Lepidoglyphus destructor, Tyrophagus putrescenciae, Cladosporium herbarum, Alternaria tenuis, Blatella germanica, cat, dog, Dactylis glomerata, Phleum pratense, Poa pratensis, Plantago lanceolata, Taraxacum officinale, Parietaria judaica, Artemisia vulgaris, Chenopodium album, Platanus, Quercus suber and Olea europaea.* Histamine dihydrochloride was used as a control positive (10mg/ml) and saline solution as negative control.

Table 1
PATIENT CHARACTERISTICS

Population	Number	Male/Female	Mean age (years)	Age range
Control	41	12/29	79±7	65–94
Asthmatics	64	21/43	72±5	65–83

Table 2
SUBSTANCE P EXPRESSION

Population	SP (pg/ml)** Mean value	SP (pg/ml) – Median
Control	39.5±41.7	17.97
Asthmatics	116.2±138.9	49.02

*Non-parametric test of Mann-Whitney **[author, please check: no * in table]**
** Statistical significance, $p = .009$

Table 3
TAS AND SOD EXPRESSION

Population	TAS(pl) mM*/** Mean value	TAS(pl) Median	SOD(gv) U/gHb*/** Mean value	SOD(gv) U/gHb Median
Control	0.91±0.10	0.93	822.9±179.5	826.20
Asthmatics	0.85±0.13	0.85	588.1±156.1	562.90

* *t* parametric test
** Statistical significance, $p = .000$

All patients were observed by a physician and performed a spirometric test using the same equipment (Vitalograph Compact) at least 6 h after the last dose of any bronchodilator. Predicted values were measured according to Knudson and colleagues [22]. Their spirometric performances were assessed by means of a computerized program according to ATS'94 criteria. The approval for analysis was determined using ATS'94 criteria; accuracy was achieved if, within the same evaluation, three curves were acceptable and reproducible.

The 41 control subjects and the 64 asthmatic subjects voluntarily agreed to provide blood samples; 30–50 ml of peripheral blood was withdrawn from the vein of the forearm.

SP was measured in serum samples by a competitive enzyme immunoassay (R&D Systems, Minneapolis, MN, USA). Within 5 min of collection approximately 500 KIU/ml of Aprotinin were added to all samples in order to avoid protein degradation. The absorbance was read at 405 nm with a wavelength correction set between 570 nm and 590 nm in a microplate reader (Behring ELISA Processor II, Dade Behring, Liederbach, Germany), the intensity of the color being inversely proportional to the concentration of SP in the sample.

Plasma was obtained to evaluate total antioxidant status (TAS) using Randox reagents according to methods that analyze the inhibitory capacity of 2,2'-azino-di-3-ethylbenzotazolina sulfonate syntheses. Superoxide dismutase enzymatic (SOD) erythrocyte was evaluated by McCord and Fridovich/Flohé and Ötting methods [23, 24].

Peripheral blood cells were stained with monoclonal antibodies anti-CD26 phycoerythrin (PE) (Immunotech, Marseille, France), and anti-CD3 phycoerythrin cyanine 5 (PECy5) (Dako, Denmark) used as per the manufacturer's specifications. Flow Cytometry (FACS) data were collected on a FACS Calibur (BD Biosciences, San José, CA, USA) and analyzed using Paint-a-gate (BD Biosciences, San José, CA, USA) software.

Serum DPPIV activity was measured by a fluorimetric assay previously described by Scharpe and colleagues [25]. DPPIV catalyses the cleavage of the fluorogenic substrate Gly-Pro-4-Me-

Table 4
DPPIV Expression

Population	DPPIV(U/L)*/ ** – Mean value	DPPIV(U/L) – Median
Control	58.6±14.3	60.88
Asthmatics	69.7±15.2	70.25

* *t* parametric test
** Statistical significance, $p = .000$

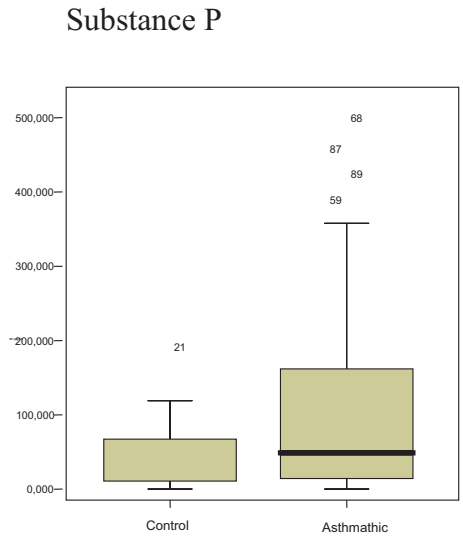

Figure 1. Substance P Expression.

2-NA, releasing a highly fluorescent molecule: 4-Me-2-NA. Substrate, Gly-Pro-4-Me-2-NA was purchased from Sigma-Aldrich, St. Louis, MO, USA. Standard solution, 4-Me-2-NA was acquired from Bachem Feinchemikalien AG, Budendorf, Switzerland. The stock solution is 50mmol/L 4-Me-2-NA in DMSO. Fluorescence was measured with a JASCO FP-777 spectrofluorimeter, with a quartz cell, at 340 nm of excitation and at 425 nm of the emission wavelengths. Serum DPPIV activity has been expressed in units/liter (U/L). One unit (U) of DPPIV activity was defined as the enzyme activity that produced 1 µmol of 4-Me-2-NA in 1 min under the reaction conditions.

Statistical calculations were performed using the SPSS 12.0 software package. The Kolmogorov-Smirnov test was used to check if variables were normally distributed. For those who had a normal distribution, the parametric t test for two independent parameters was used. Variables that were not distributed normally were evaluated using the Mann-Whitney nonparametric test. P values less than 0,05 were considered significant. Statistical comparisons were done between the two groups, controls and asthmatics. If scientifically relevant, comparisons between allergic and nonallergic participants were also done.

Results

Among asthmatic individuals, 42 subjects (65.6%) presented positive skin prick tests to common aeroallergens. Seventy percent of the patients were sensitized to house dust mites and most of them were polysensitized. As no significant differences were observed between allergic and nonallergic groups when biological parameters were analyzed, the asthmatic patients were evaluated as a group. Asthmatic patients demonstrated an increased expression of SP compared to normal control subjects ($p = .009$) (see Table 2 and Figure 1) and a decreased expression of TAS and SOD (see Table 3 and Figure 2). Asthmatic patients also demonstrated an increased expression of DPPIV compared to normal control subjects ($p = .000$) (see Table 4 and Figure 3) and increased values of CD26 and CD3CD26 without statistical significance (see Table 5 and Figure 4).

All patients were clinically stable and presented an average percentage of predicted forced expiratory volume in the first second (FEV_1) of 73.6±25.3 and median expiratory flow percentage of predict (MEF50) of 38.8±26.7.

Discussion

The increased value of SP presented in asthmatic patients when compared with normal controls confirms the role of this neurokinin in the disease. The studies were carried out in individuals older than 65 years that had had asthma symptoms for more than 30 years. SP stimulates the smooth muscle receptors inducing bronchoconstriction and narrowing of the airways. When inducing neurogenic inflammation, SP increases the production of oxygen radicals, as well as cellular activation, mainly lymphocyte activation. The asthmatic population studied presented lower values of respiratory parameters when compared with controls. In the asthmatic patients, the percentage of forced expiratory volume in the first second (FEV_1) observed was negatively correlated with SP (Pearson correlation = -0.272, $p = 0.05$), which suggests that the increase of SP in these patients may have a decisive role in the functional obstructive pattern observed.

The production of oxygen radicals enhances the bronchial inflammation of asthmatic patients and antioxidant defences are considered the main mechanisms of negative control in this process. The release of oxygen radicals is induced by SP which can be significantly increased in asthma. The significant decrease of TAS and SOD observed may affect tissue repair in asthma. During the ageing process, the antioxidant defences

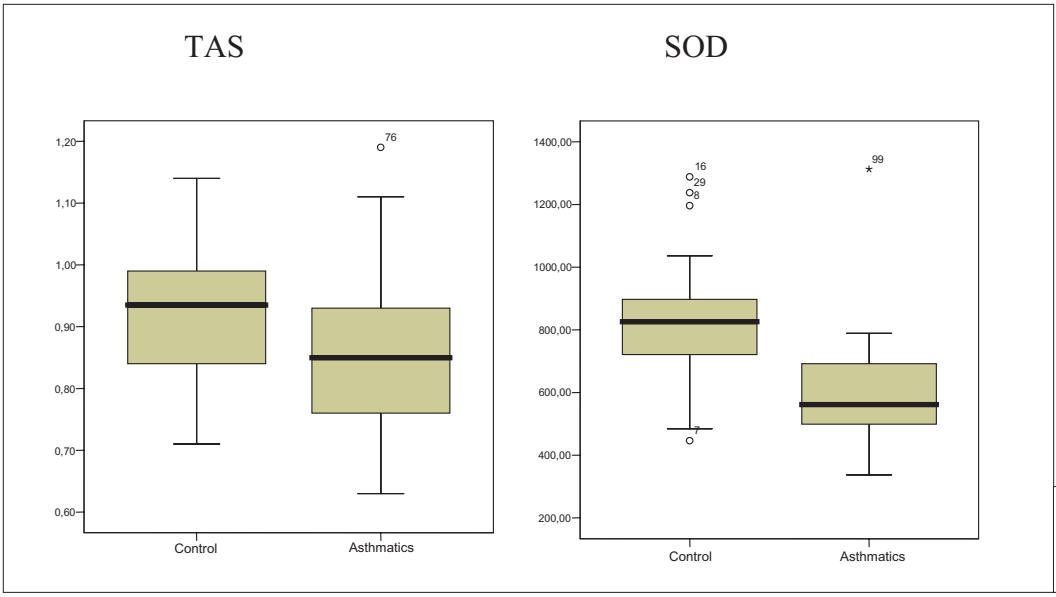

Figure 2. Total antioxidant activity in serum and superoxide dismutase expression.

Table 5
CD26 and CD3CD26 Values

Population	CD3/CD26*/** Mean value	CD3/CD26 Median	CD26*/** Mean value	CD26 Median
Control	36.2±10.6	35.70	39.4±11.4	37.55
Asthmatics	39.8±8.9	40.40	41.9±10.2	43.10

* *t*-parametric test
**No statistical significance

tend to decrease, which is associated with susceptibility to chronic inflammatory diseases. The reduction of tachykinin receptors or decrease in the neurogenic answer to mediators such as acetylcholine in the elderly, reported in some studies, does not appear to limit this neurogenic inflammation [26, 27].

The increase in CD26/DPPIV values in the asthmatic patients when compared to the control population can result from cell activation during the inflammatory process. Considering that DPPIV can be involved in the catabolism of peptides implicated in asthma pathology, the significant increase of serum DPPIV observed probably represents a negative control to limit enzymatic activity of SP and other proteases.

The enzymatic activities involved in the degradation of other tachykinins, such as the conversion enzyme of the angiotensin and the neutral endopeptidase, probably have a modulating role in the neurogenic inflammation of the airways. It was accepted that increased activity of neuropeptides or the reduction of their catabolism was associated with an increase in vascular permeability of the nasal mucous membrane with plasma transudation and inflammatory cell infiltration [28]. DPPIV seems to maintain low values up to 65 years of age [29, 21], while individuals over 65 years of age can present a sustained higher expression of this peptidase [17].

The task of the inflammatory cells in neurogenic inflammation is difficult to define and one of the main problems lies in the changes of these cells' receptor expression during the different phases of their maturation and traffic from their origin to the inflammatory injury [1]. SP can work as an inductor of mediator release from inflammatory tissues, creating a positive mechanism to continue the inflammatory process and the potential immune modulating ability. This explains the paradox observed between the relatively low concentration of SP in the nerve endings of the airways and its considerable presence in sputum and in bronchoalveolar lavage fluid. This is probably a result of local production of SP by inflammatory cells [30] which confers on this neuropeptide a crucial responsibility in airway obstruction. The local microenvironment in the different tissues seems to be an important feature for the development of SP activities. In fact, it was demonstrated that SP injection into healthy skin does not induce neutrophil infiltration, while the same procedure in inflamed skin causes neutrophil accumulation [7]. It is believed that this activity is not specific to the skin.

Bronchoalveolar lavage fluid and sputum samples collected from asthmatics after antigen challenge showed an increase in SP. The amount of SP determined in lung tissue was reduced in asthmatics and in patients who died *in status asthmaticus,* thus

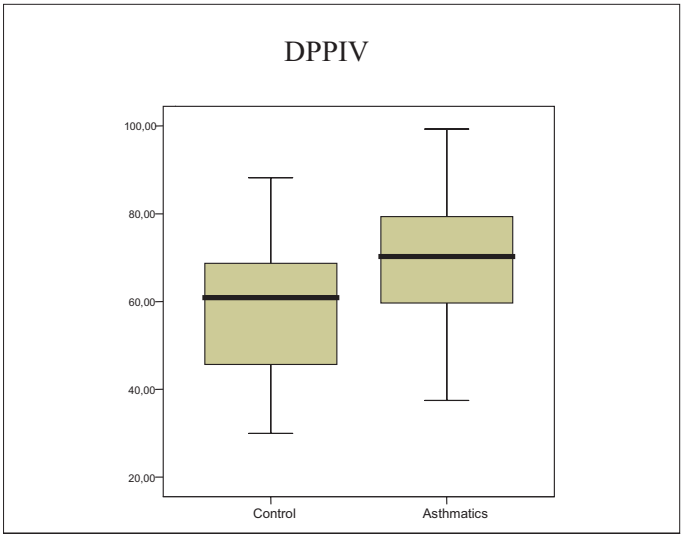

Figure 3. DPPIV expression.

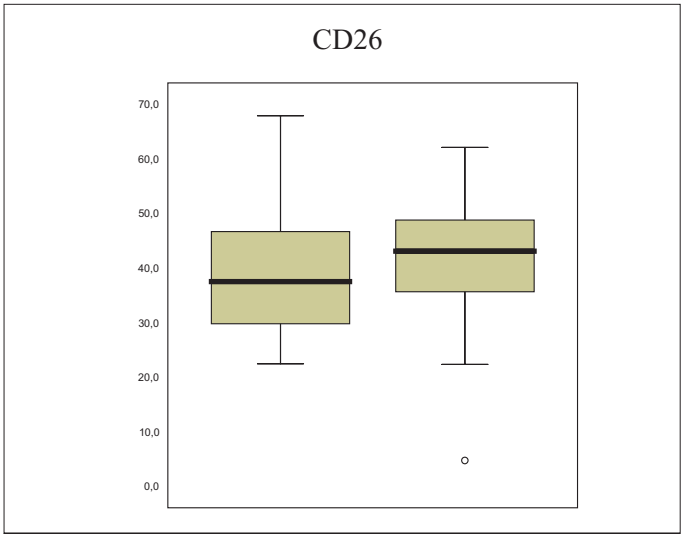

Figure 4. CD26 expression.

reflecting exaggerated release of SP in the airways of asthmatics and the consequent depletion in the lung parenchyma [1].

Within the airways, immunoreactive tachykinins are present in nerve fibers which are localized to submucosal glands, airway smooth muscle, bronchial vessels and airway epithelium. It was also demonstrated that after the elimination of cholinergic and adrenergic neural pathways, the nonadrenergic, noncholinergic (NANC) neurogenic secretory response remained, comprising approximately 40% of the total secretory response, which confirms the important role that SP assumes in respiratory pathology [10].

The sensitization to common aeroallergens observed in some asthmatic patients did not introduce significant differences in the biological markers that were analyzed. However, according to other authors, airway narrowing and the level of bronchial responsiveness are associated with atopy in adults > 65 years of age and should not be neglected [31]. In spite of the tendency of immunoglobulin E (IgE) values to decrease with age, IgE-mediated allergy can be present in 75% of elderly asthmatics [32].

The human respiratory tract epithelium can produce antioxidant species as a defence mechanism since they are consumed to face different triggers [33].The reduction of the values of superoxide dismutase has been referred to in asthma and in other chronic diseases and consequently the balance between the oxidative challenge and antioxidative defence is affected. Some asthmatic patients seem to have only a SOD reduction [34, 35]. Consistent with these observations, higher levels of ROS have already been reported in exhaled air condensates of asthmatics which are directly correlated with the severity of the disease [8, 12, 36]. These changes may occur associated with nutrient intake disturbances and chronic inflammatory disabilities. Some studies highlight a potential role of antagonists of NK-1 and of the ROS deplectors in the therapy of asthma [12].

In conclusion, the results confirm the role of SP in bronchial asthma and help to clarify the network of interactions connected with this disease. It should be emphasized that these immunoinflammatory changes are present in the elderly population with breathing pathology. The data suggest that additional therapeutic approach to this pathology should be developed.

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